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Clonal structure of invasive hoary cress (*Lepidium draba*) infestations

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Hoary cress is a perennial herbaceous weed that has invaded agricultural and natural areas of western North America. Invasions are often composed of dense patches, and it is unclear whether clonal growth via lateral rhizomes or seedling recruitment is the dominant method of patch expansion. To study the clonal structure of this invasive, six patches from three USA populations (194 ramets) were analyzed with the use of Amplified Fragment Length Polymorphisms (AFLPs). Known siblings and clones were also included to ensure sufficient variation for discrimination between clonal and nonclonal ramets. Patches had low genet/ramet ratios (mean $G/N = 0.25$) and low diversity levels (mean $D = 0.49$) compared to similar clonal studies. Single genets represented 55–85% of the ramets sampled in a patch, and the largest genet was 38 m across. Hoary cress exhibits a strong bias toward patch-size increase from clonal reproduction rather than from seedling recruitment. Results indicate that biological control methods that focus on reducing or eliminating seed production would do little to stop expansion of a patch. Despite the domination of a patch by one or a few large genets, other smaller genets are able to persist or are occasionally recruited into dense areas of a patch.

Nomenclature: Hoary cress, *Lepidium draba* L. [= *Cardaria draba* (L.) Desv.], CADDR.

Key words: Clonal reproduction, invasive weed.

Many plant species are capable of clonal reproduction through methods such as vegetative spread, production of bulbils, or asexual production of seeds. Potentially independent parts of clonally reproducing plants are known as ramets, and all ramets that originate from one zygote are collectively described as a genet. All ramets of a single genet, barring any somatic mutations, will be genetically identical. Plant species that reproduce exclusively or usually by these clonal processes tend to have lower genotypic diversity (Silander 1985), but comparisons of predominantly sexual and clonal species have revealed a smaller difference in diversity than was originally expected (Ellstrand and Roose 1987). For plant species that are both sexually and asexually reproducing, a bias toward either reproductive mode could have an influence on genetic diversity, population structure, effective population size, and evolutionary response to environmental change (Eckert 1999; Eckert et al. 2003; Hamrick and Godt 1989; Silander 1985).

Methods of assessing clonal structure in species that can spread by lateral rhizomes have improved with the development of molecular markers. Earlier studies relied on morphology to assess clonal relationship, or physical inspection of root connections between ramets, which is subject to error due to root breakage (during inspection and due to natural causes such as soil disturbance) and root grafting (connection of ramets from different genets). Molecular markers avoid these problems and have been used extensively to evaluate clonal structure of plants (e.g., Albert et al. 2003; Arens et al. 1998, 2005; Douhovnikoff and Dodd 2003; Douhovnikoff et al. 2004; Eckert et al. 2003; Kreher et al. 2000).

Hoary cress (also known as whitetop) is a perennial herbaceous weed that was accidentally imported from Eurasia to North America in the 1800s (Mulligan and Frankton 1962). This species invades pastures, crops, rangelands, and riparian areas and is considered noxious in most central and western states (USDA 2004) and some provinces of western Canada (Rice 2004). Hoary cress forms dense patches (Mulligan and Findlay 1974) in both rangeland and natural areas, but there is conflicting evidence over whether lateral rhizomes or seedling recruitment contribute more to the growth of invasive patches (a patch being defined here as a densely populated region within a population, and a population being defined as a group of plants growing closely enough to expect gene flow through pollination). Adult plants, which are self-incompatible (Mulligan and Frankton 1962), can produce thousands of seeds (Corns and Frankton 1952) with up to 84% viability after 1 yr (Bellue 1946), and these could easily be the source of patch size increases if germination and growth conditions are correct. Hoary cress seeds normally germinate in both spring and fall and can last for 3–4 yr in the soil seed bank (Bellue 1946). Alternatively, clonal reproduction may drive patch-size increases because, in the absence of competition, a single hoary cress plant can produce over 400 ramets per year from lateral rhizomes (Kirk et al. 1943), and expansion can occur at a rate of up to 3.7 m in diameter in the first year of growth (Selleck 1961). Larson et al. (2000) studied hoary cress recruitment in a xeric environment by observing *in situ* seedling germination and by excavating lateral rhizomes. The results indicated that most increases in ramet density were due to vegetative reproduction, whereas seedling recruitment, which was limited by precipitation and temperature in May, was a factor in only 2 of 8 yr. Despite these trends in recruitment, it is still unknown if hoary cress patches in the field are dominated by one extensive, clonally

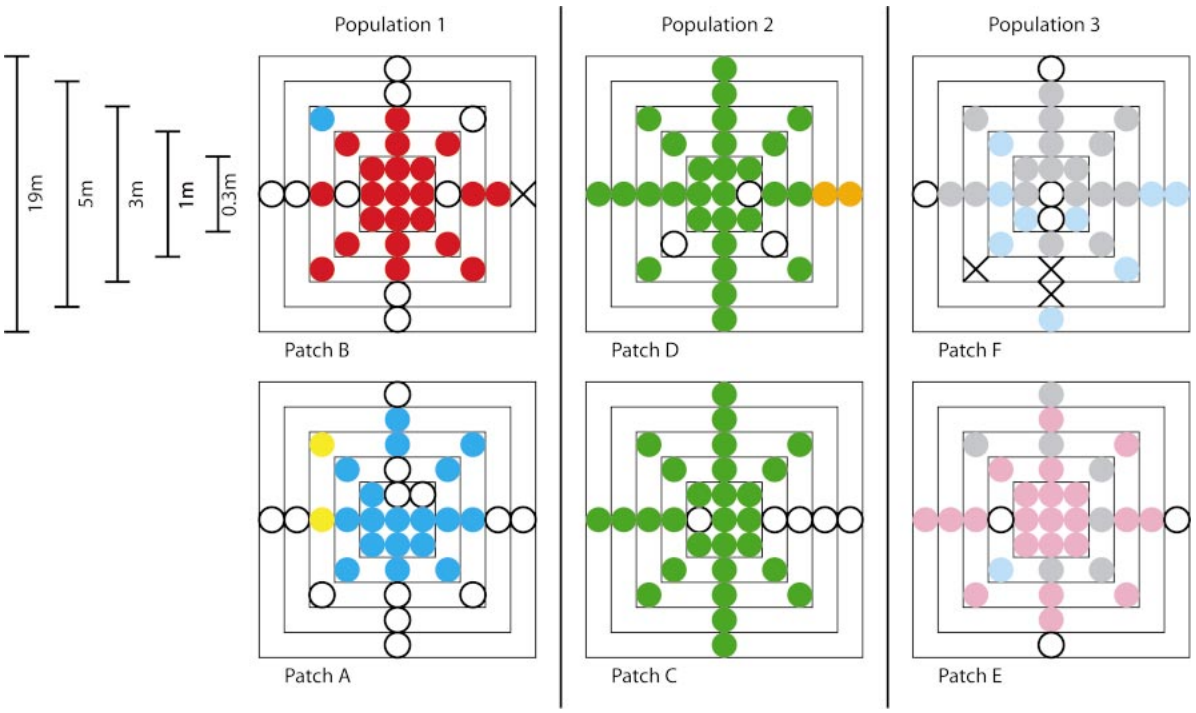

FIGURE 1. Map of hoary cress patches showing positions of sampled ramets. Ramets are represented by circles, and same-colored circles, even in different patches, are part of the same genet. Empty circles indicate ramets that were genetically distinct from all other ramets. An X indicates that the ramet did not produce data in the Amplified Fragment Length Polymorphism (AFLP) analysis. Scale of concentric sampling squares is shown on left side, and is the same for all patches.

reproducing genet, by multiple genets that produce only one or a few ramets each, or by some combination between these extremes. In this study, for collection purposes, a ramet is further defined as all plant material derived from a single shoot emerging from the soil surface.

Hoary cress is currently being investigated as a target for biological control (USDA 2002). Knowledge of its primary reproductive mode under field conditions will give us a better understanding of how the species became a successful invader, and may help determine management decisions. The objectives of this research are to use amplified fragment length polymorphisms (AFLPs) to (1) determine the clonal structure of patches of hoary cress ramets, (2) measure the maximum size of a genet, and (3) examine how measurements of population subdivision are altered when clonal relationships are revealed.

Materials and Methods

Collections

Three USA populations of hoary cress were sampled: two from Utah and one from South Dakota. Two patches (with densest portions consisting of at least 50 ramets per square meter) from each population were selected. Young leaf material, without obvious disease, from 33 ramets per patch (198 ramets total) was collected and silica dried. All vouchers are deposited at Missouri Botanical Garden herbarium (MO). Ramets were sampled from the edges and corners of concentric squares with sides of 0.3, 1, 3, 5, and 19 m (Figure 1). The sampling pattern was centered at the densest portion of a patch; thus distances between the two patches varied (from 20 to 30 m) in each population. All populations in this study are from areas that are on average warmer and wetter in May than the site used in the earlier study of seed recruitment in Baker, Oregon (Larson et al. 2000) (mean temperature of 12.2 C, mean precipitation of 33.0 mm in May, and annual rainfall of 274.3 mm). The first population in this study (containing patches A and B) was an abandoned field containing piles of imported dirt west of Salt Lake City, UT (40.81248°N, 111.95399°W, 1,287 m elevation, with means of 14.4 C for temperature and 45.7 mm of precipitation in May, annual rainfall of 411.5 mm). The second population (patches C and D) was an abandoned field between a new housing development and a major highway, also west of Salt Lake City (40.8059°N, 111.94603°W, 1,288 m elevation, same mean temperature and rainfall as Population 1). The third population (patches E and F) was in the median between a highway and railroad near Belle Fourche, South Dakota (44.66879°N, 103.83644°W, 921 m elevation, with means of 13.8 C for temperature and 76.2 mm of precipitation in May, annual rainfall of 455.4 mm). Factors such as size and age of population, soil type, or plant community were not considered in this study. The first population was chosen because in an earlier study of populations throughout the western United States and Eurasia 10 ramets from this population showed no variation in single-locus DNA sequences (Gaskin et al. 2005), suggesting that the population might be clonal. The third population was chosen because in the same study it showed one of the highest levels of genetic variation (three different DNA sequence genotypes in 10 ramets), suggesting a lower level of clonal reproduction. The second population was not part of an earlier study, but was chosen because of its high density of ramets in some areas (up to 100 ramets per square meter).

To determine the genetic distinction between clonal and

sibling ramets, 16 sibling seeds from a single inflorescence were grown. The distinction of full-sib or half-sib is unknown for these seeds, as the maternal plant was pollinated naturally in field conditions. Leaf material was collected from each of the 16 siblings, and one of these siblings was isolated and grown until 16 leaves could be collected from its multiple ramets. This plant was later removed from the soil and the roots were inspected to ensure that all ramets were from a single genet.

DNA Extraction and AFLP Analysis

Genomic DNA was extracted from approximately 20 mg of silica-dried material with the use of a modified CTAB method (Hillis et al. 1996). The AFLP method followed Vos et al. (1995) with these modifications: restriction and ligation were performed during a single step in an 11-µl reaction containing 500 ng genomic DNA, 2 U *Mse*I, 1U *Eco*RI, 1X T4 DNA ligase buffer, 0.45 U T4 DNA ligase, 0.05*M* NaCl, 0.5X BSA, 4.5 µ*M* *Mse*I adaptor, 0.45 µ*M* *Eco*RI adaptor, and H_2O. The restriction ligation was incubated at room temperature overnight, then 5.5 µl of the product was diluted to 100 µl in TE (15 m*M* Tris and 0.1 m*M* EDTA). A preselective polymerase chain reaction (PCR) was performed in a 20-µl reaction containing 4 µl of the diluted, restricted-ligated product, 1X PCR buffer, 1.5 m*M* $MgCl_2$, 0.2 m*M* each dNTP, 0.2 µ*M* of each preselective amplification primer (*Mse*I + C and *Eco*RI + A), 0.5 U Taq polymerase and H_2O. The preselective PCR consisted of 20 cycles of 30 s at 94 C, 60 s at 56 C, and 60 s at 72 C. Ten microliters of the preselective amplification product was diluted to 200 µl in TE (15 m*M* Tris and 0.1 m*M* EDTA). The selective amplification was performed in a 20-µl reaction containing 3 µl of the diluted preselective amplification product, 1X PCR buffer, 1.5 m*M* $MgCl_2$, 0.2 m*M* each dNTP, 0.1 µ*M* *Mse*I selective primer, 0.05 µ*M* *Eco* RI selective primer dye-tagged with D4 (blue), 0.5 U of Taq polymerase and H_2O. The selective PCR was 120 s at 94 C; 10 cycles of 20 s at 94 C and 30 s at 66 C (decreasing by 1 C each cycle), 120 s of 72 C; 25 cycles of 20 s at 94 C, 30 s at 56 C, and 120 s at 72 C. One microliter of each selective PCR product was combined with 0.3 µl of 600 bp size standard and 28.7 µl of deionized formamide and loaded into a Beckman Coulter CEQ 2000 fragment analyzer. Fragments were scored by the fragment analyzer software (bin width of one nucleotide, accepted peak height = 10% of second highest peak). These bins were then manually screened and scored using Genographer (ver. 1.6.0, J.J. Benham, Montana State University 2001) to visualize fragments, making this a semiautomatic scoring method, as suggested by Papa et al. (2005). Gel images were normalized across total signal to avoid errors in scoring due to band intensity. All selective primer combinations of *Mse*I + CAA, CAC, CAT, CTA, or CTA and *Eco*RI + AAG, ACC, or ACT were prescreened for eight ramets, and the two most polymorphic primer pairs were chosen (*Mse*I + CAA/*Eco*RI + ACT and *Mse*I + CAT/*Eco*RI + ACT). Sibling and clonal ramets were randomized on the Genographer image by one person and scored by another person who did not know ramet identity. Repeat runs were scored blindly and compared to original runs to calculate error rate. NTSYS-pc ver. 2.1 software (Rohlf 1992) was used to calculate the Dice (Dice 1945) similarity coefficient (identical to the Nei and Li [1979] coefficient): $2a/(2a + b + c)$, where a = number of bands present in both samples, b and c = number of bands present in only one or the other sample.

Data Analysis

The genotypic diversity found within each patch was analyzed with measurements commonly used in clonal structure studies. These included the number of distinct genotypes (genets) present in a patch (G) and the proportion of distinguishable genets (G/N) (also called the PD value in other studies, e.g., Ellstrand and Roose 1987) (where N = number of ramets sampled in the population), which ranges from $1/N$ to 1, with lower values indicating higher levels of clonal reproduction. Simpson's diversity index (D) corrected for finite sample size (Pielou 1969) was calculated with $D = 1 - \Sigma\ n_i(n_i - 1)/N(N - 1)$ for $i = 1$ to G, where n_i is the number of ramets that share genotype i. Values of D can range from 0 to 1, with higher values corresponding to greater genetic diversity. Genotypic evenness (E) (Fager 1972) was calculated, with $E = (D - D_{min})/(D_{max} - D_{min})$, where $D_{min} = (G - 1)(2N - G)/N(N - 1)$ and $D_{max} = N(G - 1)/G(N - 1)$. Values of E can range from 0 to 1, with lower values indicating that a certain genet dominates the patch, and higher values indicating that the number of ramets representing each genet is more evenly distributed.

Mantel r correlations were calculated with the program "Isolation by Distance, on the Web" (Jensen et al. 2005), comparing log-transformed geographic distance matrices and matrices of pairwise similarity coefficients within a patch. Significance of correlation ($P \leq 0.05$) was tested by using 1,000 random permutations.

Squared Euclidean distances were used to analyze molecular variance within and among patches (AMOVA) (Excoffier et al. 1992). As in Persson and Gustavson (2001), two approaches were used: (i) each ramet was included in the data set or (ii) only genets were included in the data set. Comparison of these two methods demonstrates how clonal growth may influence the estimation of population structure.

Results and Discussion

Initial scoring of the primer pairs *Mse*I + CAA/ *Eco*RI + ACT and *Mse*I + CAT/*Eco*RI + ACT by the fragment analyzer software indicated a total of 457 polymorphic fragments. These were then screened and scores were checked manually, which reduced the data to 57 and 48 (105 total) unambiguous polymorphic fragment lengths, respectively. Of the 198 ramets sampled from the six patches, 194 amplified well for both primer pairs. The remaining 4 failed repeatedly for one or the other primer pair, and those ramets were removed from the study. Similarity coefficients varied from 0.33 to 1.00 within patches (Figure 2) and 0.31 to 0.66 between populations.

Sampling Bias

A patch is not a biological unit, but is a useful term in some plant species for describing a densely populated region within a population. This study analyzed clonal structure at scales as small as 0.3 × 0.3 m; thus it was necessary to

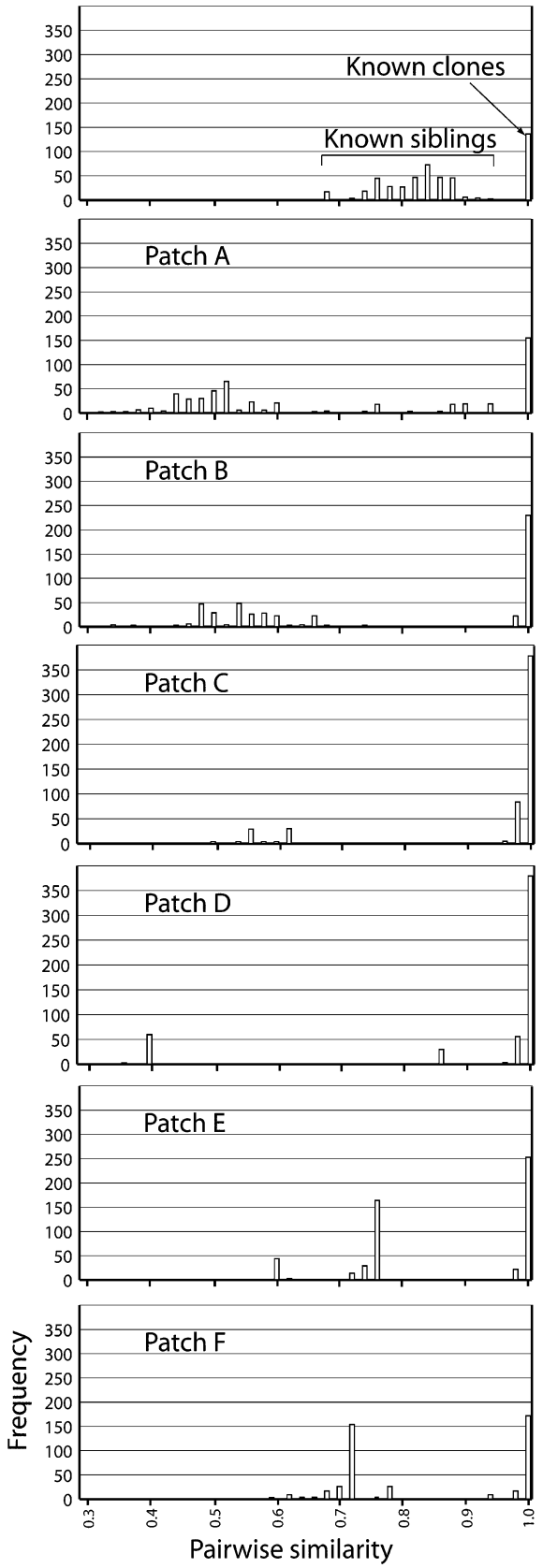

FIGURE 2. Histograms of pairwise similarity coefficients between known siblings and known clones (top chart) and within six patches of hoary cress (lower six charts).

include a densely populated area (at least 50 ramets in the innermost square meter) in each sample. This biases sampling toward ramets that are in very close proximity to other ramets, and thus the analysis does not provide information about the clonal structure of areas that are not as densely populated.

AFLP Error Rates

Knowledge of error rate in AFLP analysis is important, as errors can bias final conclusions (Bonin et al. 2004). For example, AFLP error could cause genetically identical ramets to have non-identical AFLP profiles (Douhovnikoff and Dodd 2003). To minimize the risk of this error, the AFLP process was repeated for 24 of the 194 ramets (12%) and these were re-scored blindly. Within the 105 fragments scored for each of the 24 ramets (2,520 total fragments) I found six differences between the duplicate runs, for an error rate of 0.24%. This is equivalent to an error rate of 0.2 out of 105 miscalled fragments per ramet.

Known Siblings and Clones

Another potential error in clonal analysis occurs when multilocus markers are not adequately polymorphic. Thus, ramets from different genets may exhibit identical AFLP profiles, and be mistaken as belonging to the same genet. To minimize this risk I analyzed known clonal and sibling material. None of the 16 known siblings had identical genotypes, and their similarity coefficients ranged from 0.69 to 0.95 (Figure 2). The 16 leaf samples from different ramets of a single genet all had identical genotypes (similarity coefficient = 1). The lack of identical sibling genotypes and the consistently identical clonal genotypes both indicate that the markers used were sufficiently polymorphic to distinguish sibling from clonal material. In studies where known clonal and non-clonal ramet similarity coefficients overlap, a threshold can be determined by finding the intersection of the normal frequency distribution curves (Douhovnikoff and Dodd 2003). This technique was not used here because the frequency distribution curves were not normal. Also, since the similarity coefficients of sibling and clonal material did not overlap (Figure 2), the threshold for identifying clonal material was simply set at a similarity coefficient of 1.00.

Patch Structure

A total of 157 (81%) of all 194 ramets were genetically identical (similarity coefficient = 1.00) to at least one other ramet, and these represented eight different genets (Figure 1). In contrast, only thirty-seven (19%) of all ramets were genetically distinct from all other ramets. The number of genets (G) in a patch varied from 5 to 15 (Table 1). None of the patches were composed of a single genet, but all patches did contain clonally reproducing genets (Figure 1). The amount of clonal reproduction was substantial, with a single genet representing at least 55% (and even as high as 85%) of the ramets sampled in a patch.

Many pairs of ramets in a patch had similarities in the range of the known siblings (0.69–0.95) (Figure 2), but there were also pairs of ramets in a patch that had much lower similarity coefficients (e.g., 0.33). These lower simi-

TABLE 1. Clonal structure and diversity for six patches of hoary cress from three populations.

Population	Site	No. ramets sampled	G[a]	G/N[b]	D[c]	E[d]	Mantel correlations	
							r	P <
1	Patch A	33	15	0.45	0.71	0.12	−0.69	0
	Patch B	32	11	0.34	0.53	0	−0.63	0
2	Patch C	33	6	0.18	0.28	0	−0.33	0.02
	Patch D	33	5	0.15	0.28	0.09	−0.30	0.04
3	Patch E	33	6	0.18	0.52	0.42	−0.07	0.29
	Patch F	30	6	0.20	0.62	0.56	−0.23	0.01
	Mean (*SE*)		8.17 (1.59)	0.25 (0.05)	0.49 (0.07)	0.2 (0.09)		

[a] Number of genets.
[b] Proportion of distinguishable genets.
[c] Simpson's diversity index corrected for finite sample size.
[d] Evenness.

larity coefficients (< 0.60) were found in all patches and suggest that founding events and/or subsequent recruitment involving distantly related seedlings or vegetative material, not just seed production by a single founding genet, contribute to the genetic diversity of hoary cress patches. The presence of more distantly related genets in a patch suggests that isolated patches will contain potential mates for the obligate outcrossing necessary to produce seed.

Population 1, the abandoned lot with piles of imported dirt, contained the highest mean genetic diversity (mean D = 0.62, Table 1), most likely due to the importation of soil using multiple dump truck loads, potentially from different sources.

Thirty to thirty-three ramets were sampled from each patch, but these ramets only represented 5–15 genets (Table 1). This dominance of patches by clonally reproducing genets reduces the actual population size (number of genets) 0.15–0.45 times what would be expected when compared to a visual census of individuals within populations (number of ramets). This reduced estimate of population size may increase expectations of genetic drift in populations and reduce the ability of hoary cress to adapt to environmental changes at a local level.

Some genets spanned across neighboring patches, but none were shared between populations. The maximum distance between ramets of a single genet was 38 m in Population 2 (Patches C and D, which were adjacent) (Figure 1). This was also the maximum distance between any two ramets sampled in the population, so it is possible that a genet may be even larger, given that patches can be hundreds of meters in diameter (J. Gaskin, personal observation). Hoary cress has been reported to spread 1.2–3.7 m in diameter per year, making the largest genet detected in this study 10–32 yr old. Alternatively, it is possible that soil disturbance has inflated the estimate of genet size. Hoary cress can become established not only through seed movement, but also by root fragments (Scurfield 1962), and it is possible that multiple, separated vegetative parts from a single genet could have been deposited through human disturbance across a patch area and subsequently grown independently. This would lower the maximum estimated size of a genet connected by lateral rhizomes, and also lower estimates of the age of a patch.

The proportion of distinguishable genets (G/N) in a patch varied from 0.15 to 0.45 (Table 1). Values of 1 would indicate no clonal relationship between ramets, and low values would indicate dominance by a single or just a few genets. Hoary cress genetic diversity varied in patches from D = 0.28–0.71 (Table 1). The proportion of distinguishable genets (G/N) and diversity (D) are equivalent and lower, respectively, than those found in a study of 27 clonal species of plants (Ellstrand and Roose 1987). Most of the compiled studies used isozymic data, which presented fewer characters (3–26) than typically used in AFLP or random amplified polymorphic DNA (RAPD) studies. Ellstrand and Roose (1987) found a significant correlation between number of characters scored and number of genotypes detected, which suggests that isozymic and AFLP studies of clonal species may not be comparable. A more recent comparison of clonal plant species analyzed with RAPDs (which typically present high numbers of characters) found a mean G/N of 0.44 and mean D of 0.74 (Hangelbroek et al. 2002), both higher than in hoary cress. The comparatively low G/N and D values found in the hoary cress patches both indicate the importance of clonal reproduction for this weed. Evenness in hoary cress populations varied from 0 to 0.56 (Table 1). These low values of evenness indicate that patches are dominated by a small number of genets, and genets do not share the patch evenly. Despite the dominance of genets in patches, ramets from different genets occasionally exist in the middle of a larger genet, even in the patch's densest regions (the smallest concentric square in Patches A, C, D, and F) (Figure 1). This spatial arrangement suggests that a few genets dominate a patch, but not completely, and other genets are able to persist or are occasionally recruited.

Mantel Correlations

Correlations between geographic and genetic distances in patches were mostly significant, though weak ($r < -0.69$) (Table 1), perhaps because patches are often dominated by a few genets that have spread to the edges of a patch (Figure 1). The correlations were all negative, though, indicating that a larger physical distance between ramets in a patch decreases the chance that they are part of the same genet.

Analysis of Molecular Variance (AMOVA)

The analysis of variance indicated that when all ramets were included in a survey, the percentage of molecular var-

TABLE 2. Analysis of molecular variance (AMOVA) for hoary cress patches based on Amplified Fragment Length Polymorphism (AFLP) data, considered at the ramet and genet levels.

Including all ramets	Degrees of freedom	Sum of squares	Percentage variation	P
Source of variation				
Among patches	5	1891.92	69.0	<0.001
Within patches	188	976.37	31.0	<0.001
Including only genets	**Degrees of freedom**	**Sum of squares**	**Percentage variation**	**P**
Source of variation				
Among patches	5	248.64	28.6	<0.001
Within patches	43	516.99	71.4	<0.001

iation among patches (69%, $P < 0.05$ in all cases) is much larger than that found within patches (31%) (Table 2). When only genets are counted in a population, percentage of molecular variation is almost inverted, with among-patch variation dropping to 28.6% and within-patch variation going up to 71.4%. This reversal illustrates how treating ramets as distinct individuals, when they are actually part of the same genet, can alter analyses of population structure. In an earlier study of population structure of hoary cress in the United States and Eurasia (Gaskin et al. 2005), the size of a genet was unknown, but an effort was made to collect ramets that were greater than 5 m apart, hoping that they would not be part of the same genet. Now that it is known that genets can be 38 m across or possibly larger, it is probable that many ramets collected in that study were from the same genet, and thus the level of variation among populations may have been overestimated.

Summary

These AFLP markers are clearly able to distinguish clonally related from nonclonally related hoary cress ramets. Results indicate that hoary cress patches are dominated by a small number of genets, which can be as large as 38 m across; but coexisting within these large genets are other genets that are represented by a smaller number of ramets.

The earlier study of hoary cress reproduction from seed found that seedling recruitment was a minor part of patch-size increase, especially during years of lower precipitation in the spring (Larson et al. 2000). The areas sampled in this study are both typically warmer and wetter during the time that seeds would normally germinate, yet patches are still dominated by ramets of one or a few genets, further indicating a bias toward patch-size increase from clonal reproduction, even in habitats that are more conducive to seedling recruitment.

These results clearly demonstrate that hoary cress has the ability to expand patch size in natural scenarios without seedling recruitment. Consequently, any control method that focuses on reducing or eliminating seed production, such as a biological control agent that attacks seeds, would do little to stop expansion of a patch.

The dominance of hoary cress patches by a small number of genets substantially reduces the population size from what would be expected if all ramets were genetically distinct. Measurements of population structure are also drastically altered when only genets, not all ramets, are incorporated in the analysis. Measurements of genetic diversity (*D*), which are lower than found in most other clonal plant species that have been analyzed (Hangelbroek et al. 2002), may in turn reduce our expectations of how quickly hoary cress can evolve and adapt to local environmental changes.

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